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The coming impact of gene expression profiling on the diagnosis and treatment of HCV-associated liver disease

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Abstract

Gene expression profiling allows the level of activity of thousands of genes to be monitored simultaneously. Profiling is often carried out on specialized chips or slides, which have microarrays of gene targets at predetermined addresses. In the immediate future, microarrays promise to yield new insights into hepatitis C virus (HCV) pathogenesis and to produce 'signatures' that can be used in molecular diagnostics. In the longer-term, they may aid the development of serological tests by identifying genes encoding secretory proteins produced by HCV-infected livers, and they may suggest new avenues for disease intervention by detecting genes whose products are retained in the infected liver. © 2001 Published by Elsevier Science B.V.

Keywords: Gene expression profiling; Hepatitis C virus; Messenger RNAs

1. Gene expression profiling measures the level of expression of thousands of genes

Powerful new analytical tools make it possible to compare simultaneously the levels of thousands of messenger RNAs (mRNAs) in a diseased tissue, such as a hepatitis C virus (HCV) infected liver, with the levels in healthy tissue. This is a significant breakthrough because the functional state of a cell or tissue is determined largely by the population of proteins it contains, and mRNA populations provide information, albeit imperfect information, about the proteins. In the fullness of time, user-friendly methods for defining protein populations are likely to emerge, but at the moment, the best way to assess gene expression is by examining mRNA populations. Messenger RNA populations are very useful surrogate markers of protein populations and are much easier to measure.

Several alternative approaches have been developed to measure a large fraction of the mRNA population of cells and tissues. These methods include differential display (Lian and Pardee, 1992), serial analysis of gene expression (SAGE) (Velculescu et al., 1995), and DNA microarray analysis (Phimister, 1999). In effect, these methods allow thousands of northern hybridization experiments to be done at one time. In contrast to gene mutation studies, they provide information about levels of gene *expression*, rather than about

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genetic sequences themselves. Of the various methods available, DNA microarrays are assuming a predominant role and provide the examples of gene expression profiling in this article.

2. Technical aspects of gene expression analysis

As illustrated in Fig. 1 (Buhler, 1998), to determine the gene expression profile, RNA is first extracted from a reference tissue, such as control liver, and from a second tissue, such as HCV-infected liver (step 1). The RNA is copied into fluorescently-labeled cDNA by reverse transcriptase (step 2). The cDNA from diseased tissue is labeled with one type of dye, cy-5, for example, which fluoresces red, and the cDNA from control tissue is labeled with a second dye, cy-3, for example, which fluoresces green. The two cDNA populations are mixed with each other (step 3) and washed over a microarray containing immobilized DNA probes (step 4). The fluorescently-tagged cDNAs bind to their complementary DNAs probes comprising the microarray. The microarrays contain up to 10 000 DNA probes, each located at a specified 'address' that can be 'interrogated' with a scanning device. The scanner measures the fluorescence at each spot in the array (step 5), and records the amount of light emitted from cDNAs representing the control tissue and the HCV-infected tissue. This information is analyzed by a computer (step 6) to identify genes that are over-expressed or under-expressed in the diseased tissue compared with the control. The capital cost for building both an arrayer and a scanner is now less than $60 000 (Brown and Botstein, 1999). Radiolabeled cDNAs can be used instead of fluorescently-labeled molecules, and are often used in conjunction with microarray probes attached to membranes. Arrays on membranes usually contain hundreds, rather than thousands, of probes and thus are often called 'low-density' arrays.

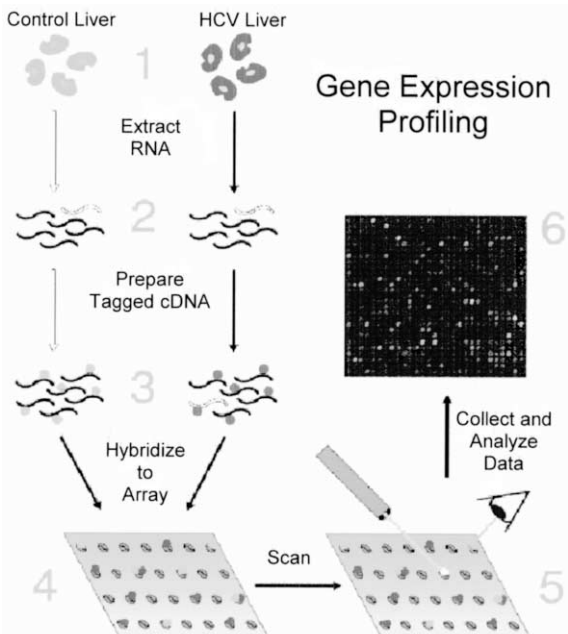

Fig. 1. Steps in gene expression profiling with a DNA microarray. RNA is extracted from control and HCV-infected liver (step 1) and reverse transcribed into fluorescently-labeled cDNA (step 2). The two differentially-labeled populations of cDNAs are mixed with each (step 3) and washed over a microarray of probes (step 4). The microarray is scanned at two wavelengths of light (step 5). Each site in the array is 'interrogated' and the signals representing the cDNAs of control vs. HCV-infected liver samples are recorded, displayed, and analyzed (step 6). Adapted from (Buhler, 1998), with the kind permission of J. Buhler.

Regardless of the type of label or microarray, the results need to be confirmed using complementary approaches. The levels of individual mRNAs can be measured by 'real-time polymerase chain reaction (PCR)' or Northern blotting. Similarly, individual proteins can be studied by immunocytochemistry and Western blotting.

The microarrays currently in use differ in the length of the immobilized DNA probes comprising the arrays. GeneChips produced by Affymetrix contain DNA oligomers about 25 bases in length generated in situ by a photolithography process, while most other DNA microarrays use DNA molecules that are hundreds to thousands of bases in length. These longer DNAs are maintained usually as a plasmid library. A large collection of 40 000 well-characterized (sequence validated) human clones is available (see, www.researchgenetics.com). To make use of such a collection, individual clones are amplified in PCR reactions to produce DNA probes for spot-

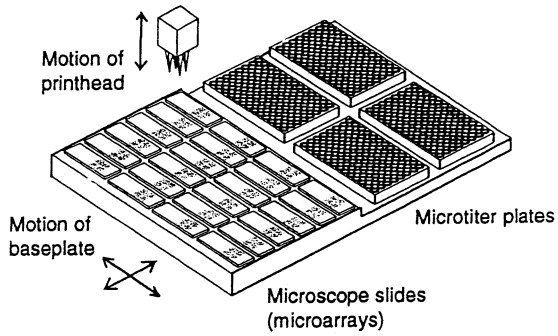

Fig. 2. Robotic arraying machine. To create a microarray, the printhead transfers a minute amount of PCR-amplified cDNAs from microtiter plates to designated positions ('addresses') on microscope slides. A computer controls the movement of the printhead and the baseplate. Reproduced from (Schena, 1996), with the kind permission of M. Schena.

ting. As illustrated in Fig. 2, PCR products can be applied robotically to a support matrix, typically a glass slide, by an arrayer (Schena, 1996). Brown and his colleagues at Stanford have been at the forefront of almost every aspect of microarray development, from conceptualization of the approach, to the construction of a machine for spotting PCR products onto microscope slides (the Arraymaker), to the design of software for data analysis. Their website, www.cmgm.stanford.edu/pbrown, provides a great deal of useful information (Brown, 1998). A second website, www.gene-chids.com maintained by Shi, provides a survey of microarray methods and provides useful links to additional sites (Shi, 2000). Optimal microarray experiments will require methods for sample preparation and RNA extraction that have not yet been fully worked out. Tissues vary in their level of ribonucleases and in their tolerance for being left at room temperature prior to freezing or RNA extraction. Madejon and his colleagues in Carreno's laboratory reported that levels of HCV RNA and a representative cellular mRNA declined in liver biopsy samples exposed to room temperature for more than 4 min, underscoring the need for efficient sample handling (Madejon et al., 2000).

3. Goals of gene expression profiling and early successes with large-scale mRNA analyses

Microarrays will have many applications in biomedical research. Three major goals are to define the gene expression signature of various conditions; to use these signatures in diagnosis and making prognostic decisions; to use profiles to guide drug discovery and to individualize patient care (see Fig. 3). For a disease with a highly variable outcome, such as HCV, it may be possible to identify a pattern of gene expression that is associated with a benign course of disease and to find pharmaceuticals, which promote this pattern of gene expression.

To date, some of the most advanced research with microarrays has been in the field of hematology. This research warrants review because it illustrates what can be expected in the field of hepatology in the near future. In a landmark study, Golub and colleagues in Lander's laboratory extracted RNA from the cells of 27 patients with acute lymphoblastic leukemia (ALL) and from 11 patients with acute myeloid leukemia (AML) (Golub et al., 1999). They then deter-

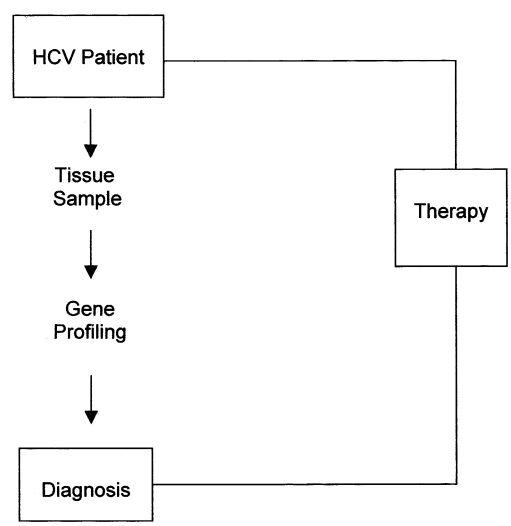

Fig. 3. Use of HCV microarrays in HCV diagnosis and treatment. In the future, gene expression analysis will provide detailed information about the type of damage that is occurring in the HCV-infected liver of an individual patient and allow the optimal treatment to be identified.

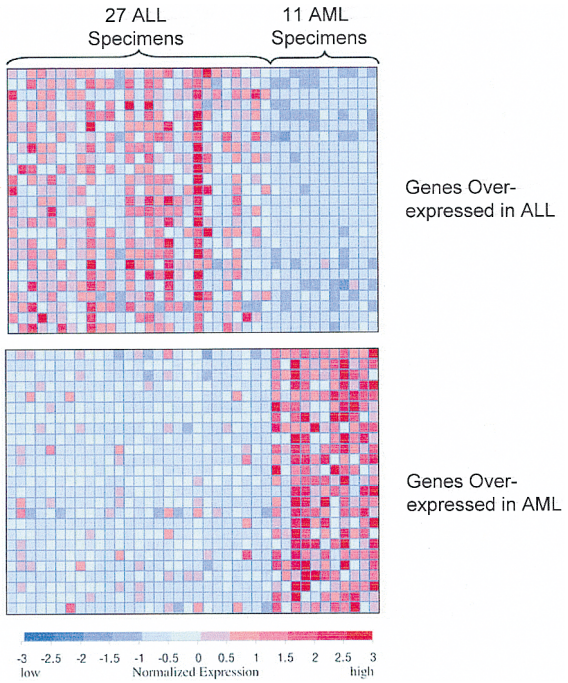

Fig. 4. Gene expression patterns of ALL and AML. This figure shows the expression pattern of 50 differentially expressed genes from 27 ALL patients and 11 AML patients: 25 genes are over-expressed in specimens of patients with ALL, and 25 different genes are over-expressed in specimens of patients with AML. Genes expressed at a low level are in blue, and genes expressed at a high level are in pink. Each column shows the expression level of the 50 genes in the mRNA extracted from an individual patient (Golub et al., 1999).

mined the expression pattern of 6817 genes. From this set of almost 7000 genes, they identified 50 that were expressed differentially in the two types of leukemia (see Fig. 4). These investigators then attempted to use the expression of these 50 genes to predict the diagnosis in a series of 34 blinded samples—24 bone marrow samples, and ten samples of peripheral blood. On the basis of gene expression patterns, they were able to predict strongly the diagnosis in 29 of the 34 test specimens (85%). In all 29 cases, the diagnosis was correct. Moreover, several of the samples that could not be identified were prepared using a method, which was different from that used to prepare the majority of the samples. In discussing the power of their molecular approach to cancer diagnosis, the investigators described a patient who had been diagnosed with AML. Based on the gene expression pattern, the specimen did not appear to be either AML or ALL, but rather appeared to be in the muscle cell lineage. The diagnosis of this patient was explored further by a variety of approaches. It was established eventually that the patient had rhabdomyosarcoma. Treatment was thus changed from that for AML to treatment for rhabomyosarcoma.

The success of the molecular approach for leukemia diagnosis suggests that gene expression profiles will also advance other forms of cancer diagnosis. However, because tissues, in general, and solid tumors, in particular, typically contain a mixture of cell types, their analysis is more complex. Botstein and his colleagues have been developing a two-step method for detecting distinctive gene expression patterns in multicellular tissues (Perou et al., 1999). In the first step, they manipulate cells in culture in order to identify 'instructive clusters of genes'. For example, in preparation for analysis of breast tumors, they exposed human breast epithelial cells in culture to a variety of stimuli, such as epidermal growth factor, transforming growth factor β, interferon α, and interferon γ, and determined the expression pattern of 5000 genes. Genes that showed similar responses to various stimuli were grouped into clusters. Several clusters of co-expressed genes were identified, including a proliferation cluster that was highly expressed in rapidly growing cell lines and certain tumors. Future studies will seek to establish correlations between clinical outcomes and gene expression patterns.

To apply Botstein's two-step approach to HCV-infected livers and to hepatocellular carcinomas, it will be necessary to identify a variety of stimuli that can be used to induce the expression of instructive gene clusters. Natural history studies of HCV patients, animal studies, and in vitro experiments can suggest what stimuli might be most advantageous to test, as illustrated in the following example. According to information compiled by Poynard and colleagues (Poynard et al., 1997), women who are infected with HCV when they are 40 years of age or less have a rate of fibrosis progression that is only about one-half that of women infected when they are older,

suggesting that high levels of sex steroids may induce the expression of genes that moderate the damaging effects of HCV. The suggestion that female hormones militate against HCV-associated liver damage is strengthened by a study of women who were exposed to HCV at an average age of 28 while they were pregnant (Kenny-Walsh, 1999). Seventeen years after infection, about 45% of the 704 women who tested positive for HCV antibodies were seronegative for HCV RNA, providing evidence of a high rate of viral clearance. Furthermore, in the 376 HCV RNA positive women in whom histological analysis was possible, only seven (2%) had evidence of fibrosis, and two of the cirrhotics consumed large amounts of alcohol. Evidence that estrogen can have anti-fibrotic effects has been obtained from animal studies. For example, estradiol blocks fibrosis in a rat model of fibrosis (Yasuda et al., 1999) (Fig. 5). Many of the anti-fibrotic effects of estrogen were reviewed recently by Bissel (1999). Taken together, studies of estrogen effects on the liver suggest that this sex hormone may not only be useful as a substance to induce the expression of 'instructive clusters' of genes to aid the interpretation of microarray data, but that estrogen may also be useful clinically—both to promote viral clearance (possibly in association with interferon) and to minimize HCV-associated liver damage in women with chronic HCV infection.

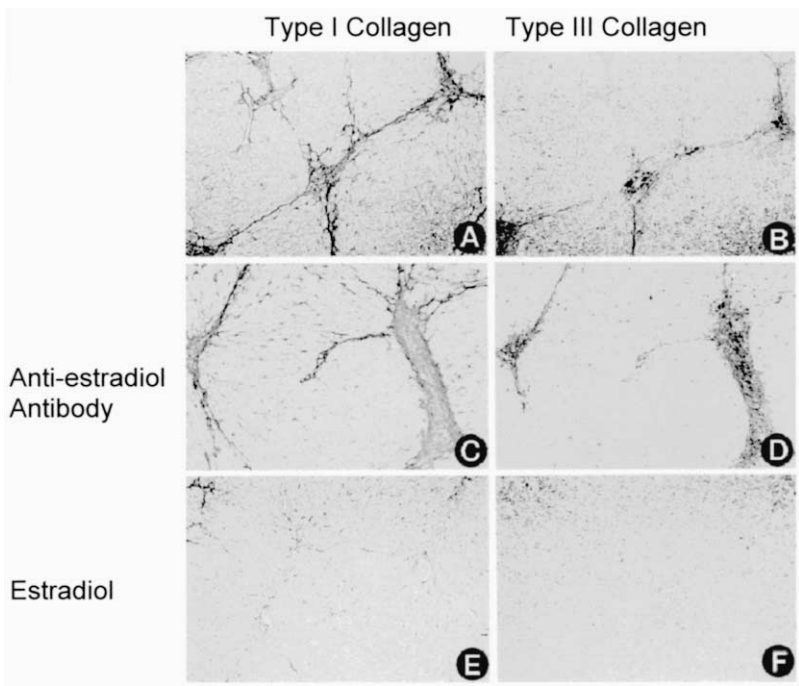

Fig. 5. Suppressive effects of estradiol on dimethylnitrosamine (DMN) induced fibrosis of the liver in male rats. Male rats were given a single injection of DMN, or DMN with anti-estradiol antibody, or DMN with estradiol and sacrificed 14 days later. Sections were immunohistologically stained for type I (A, C and E) and type III collagen (B, D and F). The livers of rats treated with DMN had high levels of type I(A) and type III(B) collagen, and those of rats treated with DMN and the anti-estradiol antibody had even higher levels (C and D), showing that extensive fibrosis occurred in the animals receiving these treatments. In contrast, the livers of rats treated with DMA and estradiol had little detectable type I or type III collagen, showing the anti-fibrotic effect of estrogen in this experimental system (E and F) (Yasuda et al., 1999).

4. Preliminary reports of liver gene expression profiles

Early reports describing the gene expression profiles of healthy, damaged, and diseased liver are beginning to appear. These studies are strong indicators of the exciting insights to expect in this field over the next few years. Several groups are pursuing studies of mRNA populations of hepatocellular carcinoma (HCC) specimens with either microarrays (Kaiser et al., 1999; Hui et al., 1999) or an alternative method, suppressive subtractive hybridization (Miyasaka et al., 1999). In a preliminary report, S. Kaiser and colleagues indicated that they found 90 genes that were up-regulated in all six HCC specimens analyzed with the Affymetrix human 6800 GeneChip In contrast, α fetoprotein was up-regulated in only a minority of the HCC specimens. Remarkably, none of the 90 genes had been associated previously with hepatocellular carcinoma. It is likely that some genes over-expressed in the cancer cells will appear in the blood. Once these products have been identified through gene expression profiling, noninvasive serological tests can be developed to detect them, potentially yielding an early screening test for HCC.

Microarrays are also finding use in studies of hepatotoxic agents (Waring et al., 1999) and viral hepatitis. McCaughan and collegues looked at gene expression patterns in control liver samples and samples from patients with end stage liver disease. Using microarrays from CLONTECH, they identified a number of genes which are up-regulated during chronic HCV infection, including the fibronectin receptor β-subunit, fibroblast growth factor receptor-l precursor, transforming growth factor-β-3, epithelial discoidin domain receptor-l, and the proto-oncogene c-jun (McCaughan et al., 2000).

Over the next few years, as the technologies of microarrays and data analysis mature, they will bring a revolution in the diagnosis and treatment of liver diseases. One of the first goals will be to define groups of genes whose expression can be grouped according to the functional and/or diseased state of the liver. These 'expression signatures' will provide the basis for developing refined and specific diagnostic tests, yielding a new, more fine-grained, classification system for liver diseases. Another important area will be the development of high-resolution screens for hepatotoxicity. Data from these screens, in conjunction with information obtained through pharmacogenomic studies, will allow therapeutic regimens to be optimized for individual patients, thereby greatly minimizing the risk of severe adverse reactions to therapeutic agents. Finally, knowledge of gene expression profiles will provide detailed information about pathways of cellular metabolism and pathogenesis. This information will guide the development of novel (combination) therapies that minimize side-effects and help to re-establish normal patterns of gene expression.

Acknowledgements

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References

Bissel, D.M., 1999. Sex and hepatic fibrosis. Hepatology 29, 988–989.

Brown, P., 1998. The Brown Lab, http://cmgm.stanford.edu/pbrown (9 May 2000).

Brown, P., Botstein, D., 1999. Exploring the new world of the genome with DNA microarrays. Nat. Genet. 21 (Suppl.), 33–37.

Buhler, J., 1998. Anatomy of a comparative gene expression study, http://www.cs.washington.edu/homes/jbuhler/research/array (24 January 2000).

Golub, T.R., Slonim, D.K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J.P., Coller, H., Loh, M.L., Downing, J.R., Caligiuri, M.A., Bloomfield, C.D., Landler, E.S., 1999. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 286, 531–537.

Hui, L., Wu, T., Hu, G., 1999. Analysis of gene expression patterns in hepatocellular carcinoma. Nat. Genet. 23, 52.

Kaiser, S., Anderson, W.F., Hwang, J.J., 1999. High-density microarray genechip analysis of altered gene expression profiles in hepatocellular carcinoma. Hepatology 30, 391A.

Kenny-Walsh, E., 1999. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. New Engl. J. Med. 340, 1228–1233.

Lian, P., Pardee, A.B., 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257, 967–971.

Madejon, A., Manzano, M.L., Arocena, C., Castillo, I., Carreno, V., 2000. Effects of delayed freezing of liver biopsies on the detection of hepatitis C virus RNA strands. J. Hepatol. 32, 1019–1025.

McCaughan, G.W., Gorrell, M.D., Bishop, G.A., Abbott, C.A., Shackel, N.A., McGuinness, P.H., Levy, M.T., Sharland, A.F., Bowen, D.G., Yu, D., Slaitini, L., Church, W.B., Napoli, J., 2000. Molecular pathogenesis of liver disease: an approach to hepatic inflammation, cirrhosis and liver transplant tolerance. Immunol. Rev. 174, 172–191.

Miyasaka, Y., Enomoto, N., Nagayama, K., Yu, S.H., Sakamoto, N., Kurosaki, M., Izumi, N., 1999. Suppression substractive hybridization identifies differentially expressed genes in human hepatocellular carcinoma. Hepatology 30, 248A.

Perou, C.M., Jeffrey, S.S., Van de Rijn, M., Rees, C.A., Eisen, M.B., Ross, D.T., Pergamenschikov, A., Williams, C.F., Zhu, S.X., Lee, J.C.F., Lashkari, D., Shalon, D., Brown, P.O., Botstein, D., 1999. Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. Proc.Natl. Acad. Sci. 96, 9121–9217.

Phimister, B., 1999. The chipping forecast. Nat. Genet. 21 (Suppl.), 1–60.

Poynard, T., Bedossa, P., Opolon, P., 1997. Natural history of liver fibrosis progression in patients with chronic hepatitis C. Lancet 349, 825–832.

Schena, M., 1996. Genome analysis with gene expression microarrays. BioEssays 18, 427–431.

Shi, L., 2000. DNA microarray (genome chip)—monitoring the genome on a chip, http://www.gene-chips.com (20 January 2000).

Velculescu, V.E., Zhang, L., Vogelstein, B., Kinzler, K.W., 1995. Serial analysis of gene expression. Science 270, 484–487.

Waring, J.F., Sullivan, J.M., Jolly, R.A., Ulrich, R.G., 1999. Gene induction/repression response profiling of hepatotoxic agents using cDNA microarrays. Nat. Genet. 23, 80–81.

Yasuda, M., Shimizu, I., Shiba, M., Ito, S., 1999. Suppressive effects of estradiol on dimethylnitrosamine-induced fibrosis of the liver in rats. Hepatology 29, 719–727.